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In mammals, the biological clock that generates circadian rhythms is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. A system for automated, long-term monitoring of circadian firing rhythms from multiple SCN neurons has been developed. In this dissociated culture system, cell interactions can be manipulated and individual rhythmic cells are accessible for further detailed study. With this system, single SCN neurons were shown to express circadian oscillations in firing rate for weeks. Despite abundant functional synapses, SCN neurons in the same culture expressed circadian rhythms of widely different phases, and even somewhat different circadian cycle lengths (periods). These data provide the strongest evidence to date that the SCN is composed of multiple circadian oscillators (clock cells).

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FINAL TECHNICAL REPORT

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(2) Publications

Welsh, D.K., Logothetis, D.E., Meister, M. and Reppert, S.M. (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* 14, 697-706.

Welsh, D.K. and Reppert, S.M. (1996) Gap junctions couple astrocytes but not neurons in dissociated cultures of rat suprachiasmatic nucleus. *Brain Res.* 706, 30-36.

(3) Introduction

Circadian rhythms in mammals are generated by a biological or circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN). The fundamental question of how the SCN circadian clock is organized at a cellular level has proven difficult to approach experimentally. This has been a serious obstacle to further work on the cellular and molecular mechanisms of circadian rhythmicity in mammals.

(4) Summary of Results

(i) Individual SCN neurons express circadian firing rhythms (Welsh et al., Neuron). Over the past few years, we have developed a method for long-term recordings of firing rates from individual cultured SCN neurons. With this technique, cells from neonatal rat SCN are cultured on fixed microelectrode plates coated with poly-D-lysine and laminin, and action potentials are recorded automatically for days or weeks from individual neurons that attach to the plate near any of the 61 microelectrodes. Our system allows for simultaneous long-term recordings of at least 8 different neurons in the same culture. Of rat SCN neurons recorded at 15 min intervals for at least 3 days, 17 out of 32 cells initially examined (53%) exhibited statistically significant circadian rhythms with periods near 24 hrs . Importantly, circadian rhythms in neuronal firing are limited to the SCN and are not found in other brain regions such as hippocampus.

(ii) SCN neurons in the same culture express independent circadian firing rhythms (Welsh et al., Neuron). Interestingly, circadian rhythms expressed by neurons in the same culture are not synchronized and appear to be oscillating independently with different periods. Simultaneous recordings of multiple cells revealed that the phases of circadian firing rhythms varies widely among cells in the same culture. Statistic analysis showed that the phase differences between clock cells are uniformly distributed between 0 and 12 hr and that there was no correlation between phase difference and distance between recording electrodes. These results suggest that different clock cells were expressing independent circadian oscillations.

In fact, long-term recordings lasting several weeks revealed that cells in the same culture could indeed express distinct circadian periods. SCN clock cells maintained circadian timekeeping in the absence of neuronal firing; after reversible blockade of neuronal firing for 2.5 days, circadian firing rhythms re-emerge with unaltered phases. These data are exciting and strongly suggest that individual SCN neurons expressing circadian rhythms (clock cells) are, in fact, autonomous, independent single-cell circadian oscillators. Thus, it appears that

intercellular communication in the SCN is not part of the circadian clock mechanism itself, but rather acts to synchronize a distributed population of single-cell circadian oscillators. The SCN, therefore, provides a unique opportunity to study a pervasive aspect of mammalian physiology and behavior at the level of a single cell.

Circadian firing rhythms of individual SCN neurons in the same culture are not synchronized, despite abundant functional synapses. This result leads to the question of whether synapses are responsible for synchronizing circadian clock cells in vivo. Synapses formed in vitro might have failed to synchronize clock cells because they were somehow inferior to those formed in vivo, either in prevalence, pattern, or neurochemical content. There is evidence for a nonsynaptic mechanism of firing synchrony among SCN neurons in slice preparations. Thus, the mechanism that normally synchronizes circadian oscillations of SCN cells in vivo, and which was evidently missing from our dissociated culture system, may well be nonsynaptic. In fact a recent study suggests that a network of SCN astrocytes coupled by gap junctions might play some role in synchronizing neuronal circadian oscillators within the SCN.

It is not known why circadian rhythms of SCN neurons are not synchronized in culture as in the SCN in situ. Study of the synchronizing mechanism is not a focus of the current application. Notably, however, the independence of oscillating SCN neurons provides a unique opportunity to study the properties of single clock cells.

(iii) Gap junctions couple astrocytes but not neurons in dissociated SCN cultures (Welsh & Reppert, Brain Res). A potential role for gap junctional coupling in SCN cultures was evaluated by introduction of the tracer molecule Neurobiotin into both neurons (n = 98) and astrocytes (n = 10), as well as by immunolabeling for specific connexins, the molecular components of gap junctions. Astrocytes were extensively coupled to each other by connexin 43-positive gap junctions in culture, but no evidence was found for coupling of neurons to each other or to astrocytes. These data support the hypothesis that neurons expressing independently phased circadian rhythms in SCN cultures (clock cells) are autonomous, single cell circadian oscillators. The data do not, however, exclude a role for glia in synchronizing neuronal clock cells in vivo.

(5) Concluding Remarks

Our knowledge of the characteristics of actual pacemaker cells in the SCN has been limited by lack of a method to identify them and to study them directly. The system developed in this laboratory of directly recording firing rate from individual SCN clock cells in culture provides an unprecedented opportunity to elucidate fundamental characteristics of the mammalian circadian clock.